Appendix A

Figure 1 - Comparison of MAPG from *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* to prevent type 1 diabetes in NOD mice.

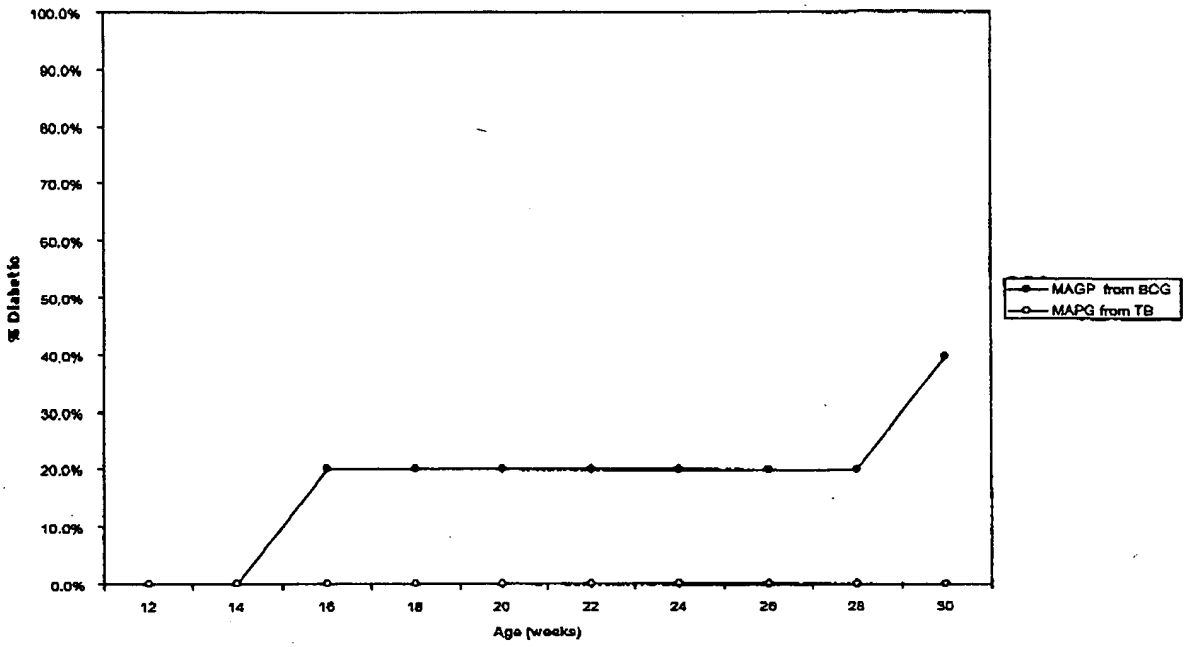
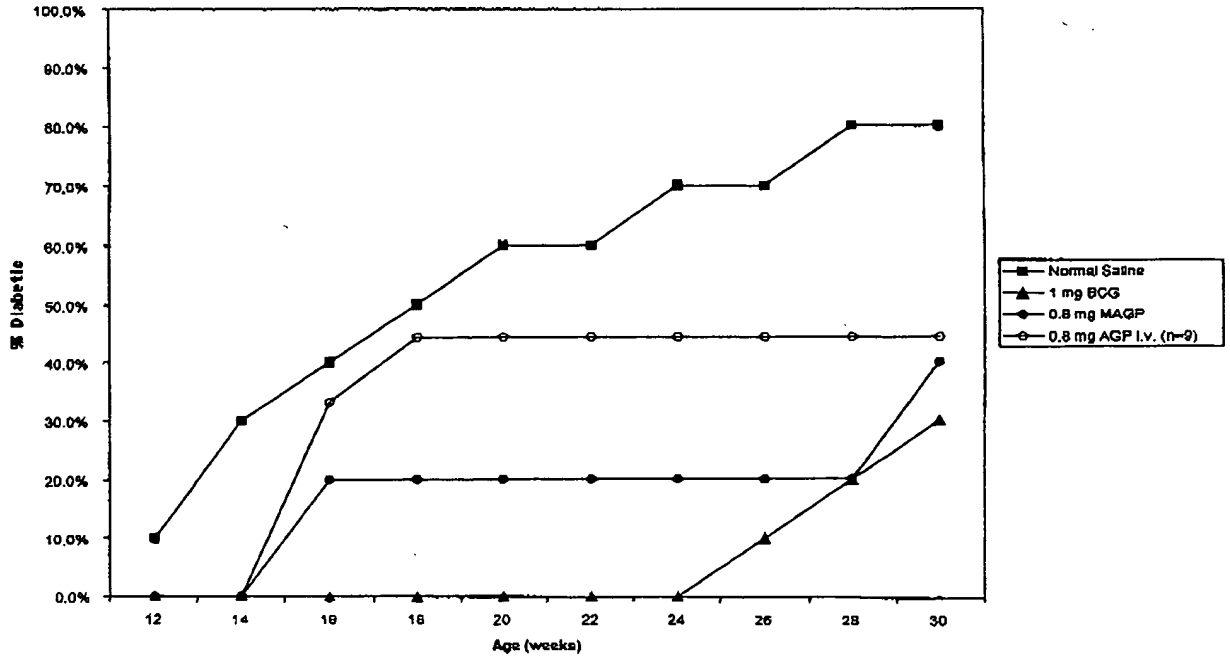


Figure 2 - Comparison of MAPG and APG from *Mycobacterium bovis* BCG to prevent type 1 diabetes in NOD mice.



Annu. Rev. Biochem. 1995. 64:29–63

THE ENVELOPE OF MYCOBACTERIA

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KEY WORDS: cell wall, arabinogalactan, mycolic acid, permeability barrier, drug resistance

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ABSTRACT

Mycobacteria, members of which cause tuberculosis and leprosy, produce cell walls of unusually low permeability, which contribute to their resistance to

0066-4154/95/0701-0029$05.00

therapeutic agents. Their cell walls contain large amounts of C_{60}-C_{90} fatty acids, mycolic acids, that are covalently linked to arabinogalactan. Recent studies clarified the unusual structures of arabinogalactan as well as of extractable cell wall lipids, such as trehalose-based lipooligosaccharides, phenolic glycolipids, and glycopeptidolipids. Most of the hydrocarbon chains of these lipids assemble to produce an asymmetric bilayer of exceptional thickness. Structural considerations suggest that the fluidity is exceptionally low in the innermost part of bilayer, gradually increasing toward the outer surface. Differences in mycolic acid structure may affect the fluidity and permeability of the bilayer, and may explain the different sensitivity levels of various mycobacterial species to lipophilic inhibitors. Hydrophilic nutrients and inhibitors, in contrast, traverse the cell wall presumably through channels of recently discovered porins.

INTRODUCTION

Most mycobacterial species appear to be saprophytic inhabitants of soil, but a few are important pathogens. *Mycobacterium tuberculosis* causes tuberculosis, a disease that still kills about 3 million people every year, mostly in the developing part of the world. Although the incidence of tuberculosis had been declining steadily in industrialized countries, this trend was reversed in the United States about 10 years ago. Even more alarming is the appearance of multiple drug–resistant strains of *M. tuberculosis* (1), a problem compounded by the availability of few drugs for the treatment of tuberculosis. Another species, *M. leprae*, causes leprosy, a chronic disease afflicting about 12 million people in the world. Finally, "atypical mycobacteria" that include *M. avium* complex, *M. kansasii*, *M. fortuitum*, and *M. chelonae*, cause opportunistic infections in immunologically compromised patients, such as AIDS patients, although they are likely to be essentially saprophytic organisms.

Mycobacteria are problem pathogens primarily because they are resistant to most common antibiotics and chemotherapeutic agents (2). Thus *M. tuberculosis* is susceptible only to aminoglycosides (e.g. streptomycin) and rifamycins (e.g. rifampicin) among antibiotics, and to fluoroquinolones among general chemotherapeutic agents. *M. leprae* is susceptible only to rifamycins, fluoroquinolones, and dapsone (a sulfonamide). Atypical mycobacteria are especially resistant, showing occasional susceptibility only to some fluoroquinolones and to clarithromycin. Thus very few general-purpose antimicrobials can be used, although several agents active specifically against *M. tuberculosis* and a few other species—including isoniazid, ethionamide, pyrazinamide, *p*-aminosalicylic acid, and ethambutol—do exist (3). However, most of these agents have little activity against atypical species (2).

Mycobacteria are also relatively resistant to drying, alkali, and many chemi-

cal disinfectants, and this makes it difficult to prevent the transmission of *M. tuberculosis* in institutions and in urban environments in general. This general resistance and the resistance to therapeutic agents are thought to be related to the unusual structure, and the resultant low permeability, of the mycobacterial cell wall.

The characteristic component of a eubacterial cell wall is the peptidoglycan, in which polysaccharide chains composed of repeating *N*-acetyl-β-D-glucosaminyl-(1→4)-*N*-acyl-muramic acid units are crosslinked by short peptide chains linked to the acid groups of the muramic acid residues. Cell walls of Gram-negative bacteria contain outer membranes outside the peptidoglycan layer, but cell walls of the usual Gram-positive bacteria lack such membranes and are largely composed of peptidoglycan, with some additional polysaccharides or polyol phosphate polymers (teichoic acids).

Mycobacteria are a group of eubacteria that belong to a larger group of Gram-positive bacteria containing GC-rich DNA, sometimes called the actinomycete line. Within this group, mycobacteria belong to one branch, often called the *Corynebacterium-Mycobacterium-Nocardia* (CMN) branch. These bacteria produce cell walls of a unique structure, sometimes called chemotype IV cell wall, containing *meso*-diaminopimelic acid as the diamino acid in the peptidoglycan. Interestingly, the muramic acid residue is *N*-glycolylated in *Mycobacterium* and *Nocardia* (4), in contrast to the *N*-acetylation found in all other bacteria. Glycolyl groups could further tighten the peptidoglycan structure by providing additional opportunities for hydrogen bonding, but the three-dimensional structure of mycobacterial peptidoglycan does not appear to have been investigated. An important feature of the chemotype IV cell walls is the presence of a unique polysaccharide, arabinogalactan (AG), which is substituted by characteristic long-chain fatty acids, mycolic acids (5–7). Mycolic acids in *Corynebacterium* and *Nocardia* contain up to about 40 and 60 carbon atoms, respectively, but those from *Mycobacterium* usually contain 70–90 carbons. In addition, several other lipid species, many of them with unusual structures, are known to exist in mycobacterial cell wall as "free" lipids, that is as solvent-extractable lipids that are not covalently linked to the AG-peptidoglycan complex (5–9). The list of such extractable lipids is becoming longer. Recent years have witnessed the discovery and characterization of trehalose-based lipooligosaccharides (LOSs) (10) and of lipoarabinomannan (LAM) (11). The purified cell wall of *M. chelonae* was found to contain conventional glycerophospholipids (EY Rosenberg, H Nikaido, unpublished observation).

Studies of the phylogenetic relationship among mycobacterial species showed, mainly by the use of 16S rRNA sequences, that the genus *Mycobacterium* consists of two subgroups, fast growers and slow growers, and that each of these groups may be subdivided into several clusters (12–14). Some of these clusters are shown in Table 1, which additionally shows the types of mycolic

Table 1 Cell wall lipids and sensitivity to inhibitors

	Lipid composition of cell wall			Susceptibility to inhibitors[a]					
	Mycolic acids[b]		Other lipids[c]	Hydrophilic		Lipophilic			
	% *trans* at proximal position of α-mycolate	Other types		NH_2OR	PNBA	Bile salts[d]	Oleate[e]	PyrB	TolB
Fast growers									
M. fortuitum	42	α', E	GPL, LOS	R		R	R(>400)	R	
M. chelonae	51	α'	GPL	R		R	R(>400)	R	
M. smegmatis	68	α', E	GPL, LOS	S		S	R(400)	R	
M. phlei	75	K, W		S		S	R(400)	S	
M. thermoresistibile	<20	α', K, M, W		S		S	S(1.6)	S	
M. vaccae	<10	α', K, W		S		S	S(3.2)	S	
M. avium	<20	K, W		R/S		S	S(1.6)	S	

Slow growers								
M. terrae	75	K, W		R	R	S	R/S	R
M. gordonae	12	M, K	SL, LOS, PDM	d	R	S	R/S	R/S
M. tuberculosis	<10	M, K	SL, PDM	S	S	S	S	S
M. bovis	<10	M, K	PGL, PDM	S	S	S	S	S
M. marinum	<10	M, K	PGL, PDM	R	d	S	S/R	S
M. kansasii	<10	M, K	GPL, LOS, PDM	d	R/S	S	S	S
M. szulgai	?	M, K	LOS	S	R			
M. malmoense	?	?	LOS	d	R		S	S
M. avium complex	18	K, W	GPL	d	R	d[f]	R[g]	R
M. leprae	?	K	PGL, PDM					

[a] The susceptibility data are from Reference 15, unless indicated otherwise. PNBA, p-nitrobenzoic acid; PyrB, pyronine B; and TolB, toluidine blue. R, S, and d refer to resistance, sensitivity (no growth in the presence of inhibitor), and different responses depending on the particular strains, respectively.

[b] Two lines of data on mycolic acids are shown. The left column shows the percentage fraction of α-mycolates with *trans* double bonds or cyclopropane groups at the proximal position ("Y" of Figure 3). These values were estimated from the analytical data of Kaneda et al (15b). The right column shows the other mycolate classes (described below in Figure 3) known to be present (7). E, M, K, and W stand for epoxy-, methoxy-, keto-, and wax-ester mycolate, respectively.

[c] Other extractable lipid species known to be present (8, 9). GPL, glycopeptidolipids; LOS, trehalose-containing lipooligosaccharides; SL, sulfolipids; PDM, phthiocerol dimycocerosate; PGL, phenolic glycolipids.

[d] Measured by growth on MacConkey agar (without crystal violet), which contains 0.075% bile salts. Data on slow growers are from ref. 16.

[e] Data on slow growers are from ref. 17. Numbers in parentheses are MIC_{40} values (in μg/ml) from ref. 18.

[f] Among *M. intracellulare* and *M. avium*, 45% and 4% respectively grew on this medium.

[g] *M. intracellulare* is resistant, but *M. avium* shows R/S phenotype.

acids present as well as the characteristic free lipids described so far. Table 1 also shows susceptibility of various species to general inhibitory agents, characteristics that suggest the degree of permeability of the cell wall (see below). It is clear that certain lipids are limited to related groups of organisms. For example, sulfolipids are essentially limited to *M. tuberculosis*. α′-Mycolates occur only among the fast growers. On the other hand, some lipids appear to be widely distributed, such as LAM and cord factors. In the following sections of this review, we describe the structures of the various lipid components and try to relate them to the probable molecular architecture of cell wall. Possible functions and biosynthesis of the various components are also discussed.

ULTRASTRUCTURAL FEATURES

M. tuberculosis is a rod-shaped bacillus of 1–4 × 0.3–0.6 μm (15a, b), although other mycobacterial species can occur as much shorter cocco-bacilli or curved rods. The envelope of mycobacteria consists of the plasma membrane and the wall. Since it remained difficult, until recently (19), to separate physically and study those independently, an important theme of ultrastructural investigation has been attempts to relate visible structure to chemical identity (reviewed in 20). The most interpretable images of the mycobacterial cell envelope were obtained from ultrathin sections of embedded bacteria. Recent investigation using freeze-substitution methods (21) produced images that are in accord with earlier ones (22) (Figure 1) and demonstrate that the cell wall is composed of an inner layer of moderate electron density, a wider electron-transparent layer, and an outer electron-opaque layer of variable appearance and thickness. The inner layer probably contains peptidoglycan, in that its moderate electron density is consistent with a product containing carboxyl groups that bind metal ions (23). The electron-transparent layer appears to be the hydrophobic domain of the cell wall, which is dominated by the mycolate residues covalently bound to the AG (see below). This layer has a thickness of about 9–10 nm (20), and thus is much thicker than the 4–4.5 nm deep layer present in the cytoplasmic membrane (Figure 1). In both cases, the transparency is caused by the failure of the water-soluble stains to penetrate into these hydrocarbon-rich regions. The nature of the outermost electron-opaque layer of the wall was debated for some time. The layer varies in thickness (from negligible to considerable), electron density, and appearance (fibrillar, granular, or homogeneous), which is attributable to differences among species, in growth conditions, and in preparation methods for microscopy. The dye ruthenium red allowed this layer to be consistently visualized (24), suggesting that a minimal structure is probably negatively charged head groups of lipids. In some species, there may be additional carbohydrate material, such as LAM, which contains phosphate and succinyl groups, and the capsular polysaccharides—glucans, mannans, arabi-

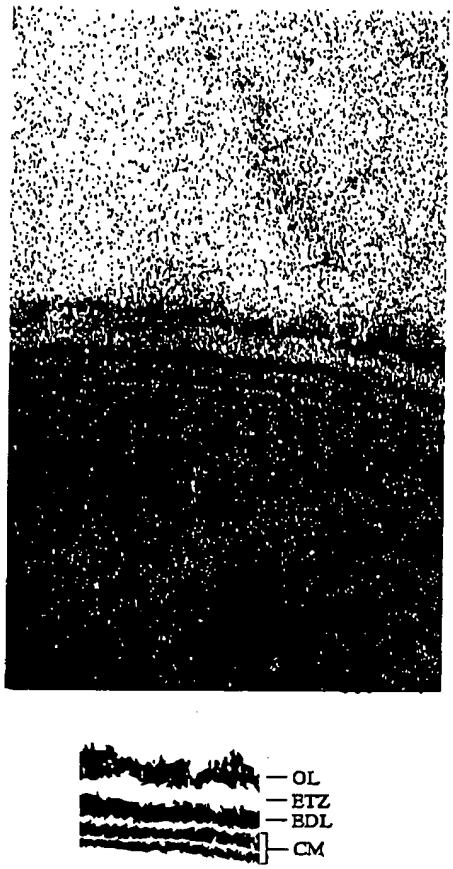


Figure 1 Appearance of mycobacterial envelope in thin sections. *a.* Electron micrograph of envelope and part of cell contents of *Mycobacterium phlei* 425. Cells were fixed by freeze-substitution (21) to optimize preservation of structure and to reduce extraction of lipid components by solvents used in processing. (Photograph reprinted from 20 with permission of the publisher.) Scale bar indicates 30 nm. *b.* Diagram showing interpretation of image shown in *a* in terms of layer structure described in text. Thickness of layers is enlarged about fourfold compared with the photograph. OL, outer layer; ETZ, electron-transparent zone; EDL, electron-dense layer; CM, cytoplasmic membrane. (Modified from Figure 15 of Ref. 21.)

nans, or other heteropolysaccharides. Proteins may also contribute to the binding of stains.

An important observation is that mycobacterial cell wall proper can be cleaved by the process of freeze-fracture. The presence of this fracture plane strongly supports the concept that the wall is constructed basically as a lipid bilayer (20).

CELL ENVELOPE COMPONENTS: STRUCTURE

Plasma Membrane

The appearance of the mycobacterial plasma membrane is not symmetrical in cells carefully fixed from a viable state, in that the outer, electron-dense layer is thicker than the inner layer in thin sections (20, 21) (Figure 1). There is cytochemical evidence that the "extra" thickness is associated with carbohydrate (25), and this suggests that the phosphatidylinositol mannosides (PIMs) (see below) are preferentially located in the outer leaflet.

PIMs The phospholipids in mycobacterial envelopes are almost invariably derivatives of phosphatidic acid. The most common are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, and phosphatidylinositol and its mannosides (PIMs). The PIMs are restricted to the bacteria of the actinomycete line (8). They are major plasma membrane components and also form the lipid base of LAM and lipomannan. The early use of ^{1}H-NMR (26) clearly established that the glycerol phosphate moiety was attached to the L-1 position of the *myo*-inositol ring and that the mannose residues were glycosidically linked to the 2 and 6 positions. The structures of each of the higher homologs, PIM_3, PIM_4, PIM_5, and PIM_6, have been established (27). They contain a single α-D-mannopyranosyl group at the 2 position, and a mannose oligosaccharide, for example [α-D-Man*p*-(1→2)-]$_2$-[α-D-Man*p*-(1→6)-]$_2$-α-D-Man*p* in PIM_6, at the 6 position (27). The PIMs, notably PIM_2 and PIM_5, contain acyl functions other than those of the diacylglycerols (28, 29).

OTHER COMPONENTS Other components associated with the plasma membrane include a number of polyterpene-based products thought to be associated with protection against photolytic damage, such as the carotenoids, and the menaquinones that are involved in electron transport (8). The carotenoids of mycobacteria are responsible for the characteristic yellow-orange color of photochromogenic mycobacteria such as *M. gordonae* and *M. kansasii*.

The known glycosylphosphopolyprenols of mycobacteria, presumably involved in cell wall biosynthesis, are also believed to be plasma-membrane-associated. The polyprenols in these are decaprenol and octahydroheptaprenol,

rather than the undecaprenol found in most common bacteria. The compounds isolated thus far are β-D-mannopyranosyl phosphodecaprenol (30), β-D-mannopyranosyl phosphooctahydroheptaprenol (31), β-D-arabinofuranosyl phosphodecaprenol (32), and 6-*O*-mycolyl-β-mannopyranosyl phosphooctahydroheptaprenol (32a).

The Cell Wall Skeleton

The covalently linked skeleton of cell wall (36) may be described as a peptidoglycan to which are linked polysaccharide side chains esterified at their distal ends with mycolic acids, that is the mycolyl-AG-peptidoglycan complex, 40% of which corresponds to lipids in the form of mycolic acids (33). The peptidoglycan contains peptide side chains consisting of L-alanyl-D-isoglutaminyl-*meso*-diaminopimelyl-D-alanine, in which the diaminopimelic acids are amidated (4). This type of peptidoglycan, type A1γ (34), is one of the commonest found in bacteria. The mycobacterial peptidoglycan, however, differs in two ways from the common type, in that muramic acid is *N*-glycolylated as described above and that the crosslinks include bonds between two residues of diaminopimelic acid as well as between diaminopimelic acid and D-alanine (35).

MYCOLYL-AG It was known already in the 1930s that the major wall polysaccharide of *M. tuberculosis* is a serologically active branched-chain AG, with the arabinose residues forming the nonreducing termini of the chains. The presence of a similar AG was shown in some seven species of mycobacteria (36). It was also demonstrated early that AG is attached to peptidoglycan through a phosphodiester link to position 6 of about 10–12% of the muramic acid residues (36). Liu & Gotschlich (37) obtained muramic acid 6-phosphate from a variety of Gram-positive bacteria, including *M. smegmatis*. There was much uncertainty, however, about the structure of the galactan component of AG.

More recent work established that the AG is rather unique in the nature of its component sugars as well as its overall structure (38) (Figure 2). Partial depolymerization of the per-*O*-alkylated polysaccharide and analyses of the generated oligomers by gas chromatography-mass spectrometry (GC-MS) and fast atom bombardment-mass spectrometry (FAB-MS) established that: (*a*) within AG, all Ara and Gal residues are in the furanose form; (*b*) the nonreducing termini of arabinan consist of a branched hexaarabinofuranosyl structure [β-D-Ara*f*-(1→2)-α-D-Ara*f*]$_2$-3,5-α-D-Ara*f*-(1→5)-α-D-Ara*f*; (*c*) the majority of the arabinan chain consists of 5-linked α-D-Ara*f* residues with branching introduced by 3,5-α-D-Ara*f* residues replaced at both branch positions with 5-α-D-Ara*f*; (*d*) the arabinan chains are attached to the galactan

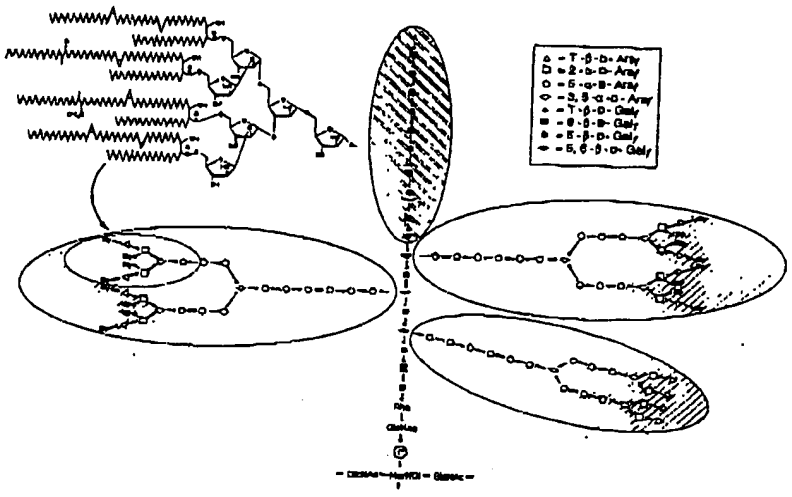
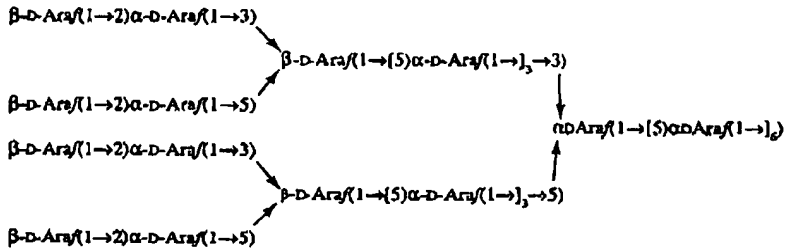


Figure 2 Chemical structure of the mycolyl-AG peptidoglycan complex of mycobacteria. The largest fragment of arabinan and galactan isolated to date are within the ellipses.

core through the C-5 of some of the 6-linked Gal*f* units; (*e*) the galactan region consists of linear alternating 5- and 6-linked β-D-Gal*f* residues; (*f*) the galactan of AG is linked to the C-6 of some muramyl residues of peptidoglycan via the diglycosylphosphoryl bridge, L-Rha*p*-(1→3)-D-GlcNAc-(1→P) (39); and (*g*) the mycolic acids are located in clusters of four on the terminal hexaarabinofuranosyl units, but only about two thirds of these arrangements are mycolated (40).

Recently, a family of arabinases and galactanases secreted by a *Cellulomonas* species was used to degrade base-solubilized AG from *M. tuberculosis* (41). The major Ara-containing degradation products were the hexaarabinofuranoside mentioned above and a linear disaccharide, α-D-Ara*f*-(1→5)-D-Ara*f*. The linear galactan backbone was degraded into cyclic oligosaccharides of the structure [5)-β-D-Gal*f*-(1→6)-β-D-Gal*f*-(1→]$_n$. More recently, oligosaccharide fragments containing up to 23 Ara residues were obtained by gentle acid hydrolysis of the per-*O*-methylated AG, and the molecular weights and alkylation patterns were determined by FAB-MS (GS Besra et al, submitted for publication). The extended nonreducing ends of the arabinan were thus shown to consist of the following unit (Figure 2):



Three such arabinans appear to be attached to the homogalactan (Figure 2). Using the same approach, an extended stretch of the galactan was also isolated consisting of 25 Gal residues, β-D-Gal*f*(1→5)-[β-D-Gal*f*(1→6)-β-D-Gal*f*(1→5) →]$_{12}$, devoid of any branching, demonstrating that the points of attachment of the arabinan chains to galactan are close to the reducing end of galactan, which itself is linked to peptidoglycan via the linker disaccharide-phosphate, mentioned above (Figure 2).

MYCOLIC ACIDS Mycolic acids are high-molecular-weight α-alkyl, β-hydroxy fatty acids, present mostly as bound esters of AG, where they appear primarily as tetramycolylpentaarabinosyl clusters (Figure 2), but also in extractable lipids, mainly as trehalose 6,6′-dimycolate (cord factor). Owing to the presence of 3-hydroxy group, pyrolysis of mycolic acids releases the part of the main chain distal to the C-3, producing "meromycolic acid." Their structures can thus conveniently be discussed by separating the molecule into meromycolate moiety and the α-branch (5).

Mycobacterial mycolic acids are distinguishable from those of other genera (such as *Corynebacterium, Nocardia,* and *Rhodococcus*): (*a*) They are the largest (C_{70} to C_{90}); (*b*) they have the largest α-branch (C_{20} to C_{25}); (*c*) in the main chain (the meromycolic acid moiety), they contain one or two groups—which may be double bonds or cyclopropane rings—that are capable of producing "kinks" in the molecule; (*d*) they may contain oxygen functions additional to the β-hydroxy group; and (*e*) they may have methyl branches in the main carbon backbone. The entire structural spectrum of mycolic acids was resolved in the 1960s (5) through the use of MS, NMR, and infrared spectroscopy (IR). The α branch, except for chain length, was found to be consistently conserved among the family of mycolic acids. Structures of various types of mycolic acids are summarized in Figure 3.

The totally saturated and fairly long (typically 24-carbon) structure of the α-branch as well as the exceptional length of the meromycolic acid chain (typically almost 60-carbon) should favor strongly the regular, parallel packing of the hydrocarbon chains of mycolic acid. On the other hand, the oxygen

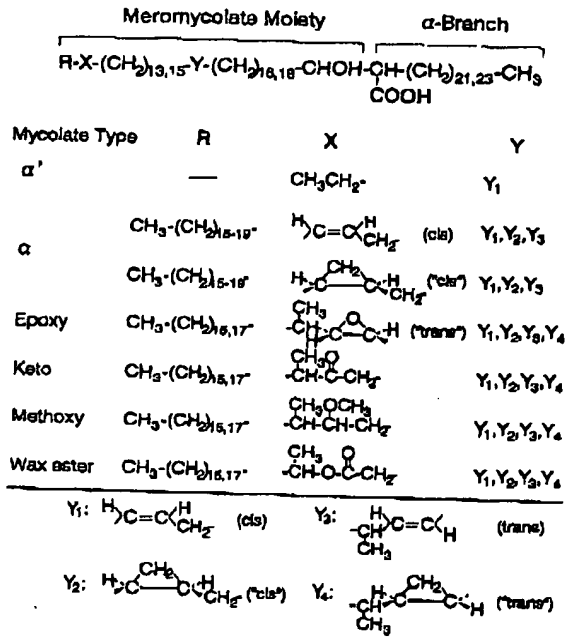


Figure 3 Structures of mycolic acids from mycobacteria. Modified from Ref. 7. In some compounds, the methyl branch in Y_3 is reported to be on the proximal side of the double bond (5).

functions, as well as *cis* double bonds and *cis* cyclopropane structures, are expected to modulate this tight packing by producing kinks in the chains. Interestingly, the packing-disruptive groups are located farther away from the carboxyl end of the molecule. Thus, all of the oxygen functions (except the β-hydroxy group) are located at position X of Figure 3, usually more than 35 carbons away from the carboxyl end of the molecule. Furthermore, the *cis* structures at the proximal position Y are frequently converted into less disruptive structures such as *trans* double bonds and *trans* cyclopropane (See Table 1); studies with conventional lipids showed that the latter structures do not produce kinks in the chain and do little to prevent the tight packing of membrane lipids (42). These points are discussed below in connection with the organization of cell wall lipids.

Lipoarabinomannan (LAM)

It was known early that mycobacteria also contained "soluble," immunologically active arabinomannan (43). Misaki et al (44) showed in 1977 that this polysaccharide from *M. tuberculosis* contained an α(1→6)-linked D-Man*p* backbone to which were attached short side chains of α(1→5)-linked D-Ara*f* residues. However, their analysis was performed on alkali-treated samples, and the finding (45) in 1970 that some of the arabinomannan from *M. tuberculosis* was acylated by palmitic and tuberculostearic acids did not attract much attention until systematic study of the native arabinomannan was undertaken in 1986. Such a study (11) showed that LAM contained glycerol, inositol, and phosphate, in addition to arabinose, mannose, lactate, succinate, palmitate, and tuberculostearate, which were identified earlier. The situation was similar with lipomannan, which is essentially LAM without an arabinose-containing side chain. The phosphate was shown to occur in the form of an alkali-labile phosphatidyl*myo*inositol unit, containing the two fatty acid residues. Thus both LAM and lipomannan were the first prokaryotic versions of the membrane components anchored via phosphatidylinositol (46), a class of compounds that occur frequently in animal cells; LAM and lipomannan are multiglycosylated extensions of PIMs (47).

Detailed structural analysis of LAM has resulted in recognition of two distinct arabinan arrangements occupying the terminal end: branched hexa arabinofuranosides with the structure, [β-D-Ara*f*-(1→2) -α-D-Ara*f*]$_2$-3,5-α-D-Ara*f*-(1→5)-α-D-Ara*f*, similar to that in AG, and a linear β-D-Ara*f*-(1→2)-α-D-Ara*f*-(1→5)-α-D-Ara*f* (48). In addition, in the case of LAM isolated from strains of *M. tuberculosis*, these two types of Ara termini are extensively capped with Man*p* residues, a product now termed ManLAM (49–51). A version of LAM, as isolated from a rapidly growing *Mycobacterium* sp. and *M. smegmatis*, is devoid of Man caps and is called AraLAM (51). It is partially capped with inositol-P residues (52). The structures of various types of LAM are schematically shown in Figure 4.

The Extractable Lipids of Cell Wall

The search for dominant antigens on the surfaces of various mycobacteria, especially "atypical" (or nontuberculous saprophytic) mycobacteria, was stimulated by the infections caused by these bacteria in immunocompromised patients (53). This led to the definition of a remarkable array of cell wall glycolipids (8, 9). We describe below the major classes of such extractable glycolipids—LOSs, phenolic glycolipids (PGLs), and glycopeptidolipids (GPLs)— as well as other classes of free lipids (See Table 1).

LOSs Members of the LOS class of glycolipids were first found in *M. kansasii* and later in *M. malmoense, M. szulgai, M. gordonae,* and *M. butyricum* (54).

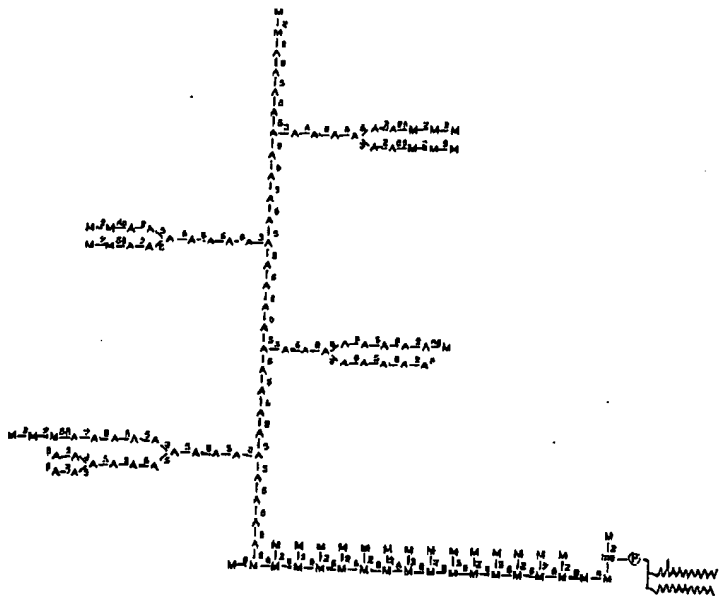


Figure 4 An attempt to present a composite structure of ManLAM from *M. tuberculosis* (Erdman strain) based on the recognition of certain small motifs and on the relative amounts of the various arabinose and mannose units in their different linkages.

Eight such glycolipids are present in smooth variants of *M. kansasii* (10). They are composed of variable residues of xylose, 3-*O*-methylrhamnose, fucose, and a novel *N*-acylamino sugar (*N*-acylkansosamine) linked to a common tetraglucose core, which itself contains an α,α′-trehalose moiety at the end (Figure 5). The terminal glucose residue of the α,α′ trehalose unit is usually acylated at positions 3, 4, and 6 by 2,4-dimethyltetradecanoic acid residues ("R" in Figure 5). The LOS from *M. gordonae* contains 6′-*O*-methyl-2,3,4,6-tetraacyltrehalose structure at one end (55).

At about the same time as the discovery of *M. kansasii* LOS, somewhat different trehalose-based LOS was discovered in *M. smegmatis* (56). The oligosaccharide unit of the *M. smegmatis* LOS contains only D-glucose residues, although two of them are pyruvylated. Furthermore, the acylation occurs on both glucose residues of the trehalose moiety, at 4′ and 6 positions (57). More recently, LOS of *M. fortuitum* biovar. *fortuitum* was found to have features of both the *M. kansasii*-type LOS and *M. smegmatis*-type LOS, in that

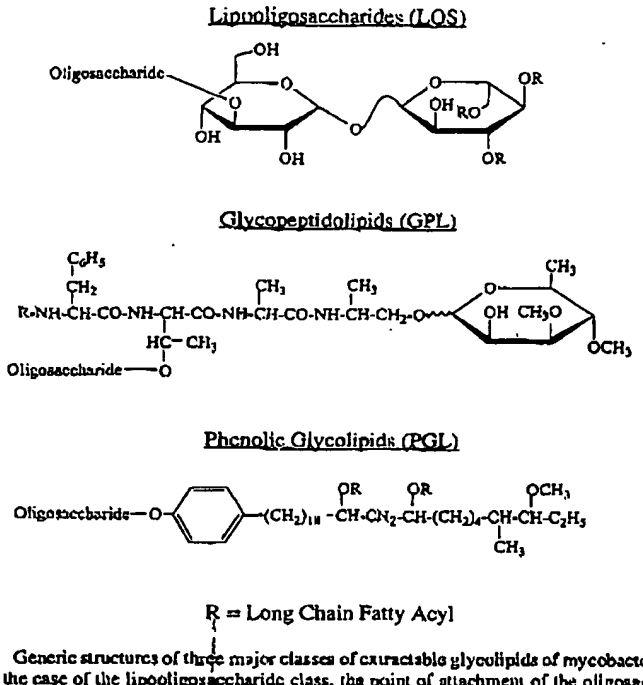


Figure 5 Generic structures of three major classes of extractable glycolipids of mycobacterial cell walls. In the case of the lipooligosaccharide class, the point of attachment of the oligosaccharide unit may also be the 4- or the 6-OH group of the acyltrehalose unit.

the acylation occurs both on the 3, 4, and 6 positions of the terminal glucose and on the 2′ position of the subterminal glucose residue of the trehalose unit (58). The chemistry and basis of the antigenicity of LOSs and their association with variable colony morphology in mycobacteria have been reviewed (54).

PGLs The second class of glycolipids is more correctly termed glycosylphenolphthiocerol dimycocerosates, although the term PGL is generally used (8, 9, 59–61). This class includes "mycoside A" of *M. kansasii*, "mycoside G" of *M. marinum*, and "mycoside B" of *M. bovis* in the earlier literature. Their structure (Figure 5) is characterized by a very large hydrophobic moiety, containing a C_{36} phenolic diol substituted by two molecules of typically C_{34} fatty acid, mycocerosate. The oligosaccharide part contains from one to four sugar residues, and the sugars are usually not very hydrophilic, consisting often of deoxy sugars that are multiply *O*-methylated.

GPLs Another class of mycobacterial glycolipids, GPLs ["C-mycosides" in earlier literature (62)], has been reviewed (54). As shown in Figure 5, the core head group is a short peptide, D-Phe-D-*allo*Thr-D-Ala-L-alaninol, and the alaninol is substituted by a 3,4-di-*O*-methyl-L-rhamnose. The hydroxyl group of the D-*allo*threonine residue carries an oligosaccharide substituent; its most proximal portion is usually α-L-rhamnopyranosyl-(1→2)-6-deoxy-L-talopyranose. The amino group of D-phenylalanine residue is substituted by a fatty acid residue.

GPLs are the major cell surface antigens of the *M. avium, M. intracellulare, M. scrofulaceum* group (MAIS), and they can be subdivided into 31 distinct serotypes based on the serospecific GPLs. The structures of the GPLs from 12 of these serovars have been completely defined (54). Although the most proximal sugars were common as described above, the nonreducing terminal sugars of the oligosaccharides were highly variable, containing an array of novel amido sugars, branched-chain sugars, sugar acids, and pyruvylated sugars. The use of monoclonal antibodies to the individual serovars of the *M. avium* complex in conjunction with semisynthetic neoantigens containing some of the precise terminal sugar combinations has established the antigenically dominant epitopes of individual serovars (63).

GPLs also constitute a major cell surface component in some fast growers, such as *M. chelonae* and *M. fortuitum* (Table 1). Recently, the structures of the major GPLs from *M. fortuitum* biovar. *peregrinum* have been established (64, 65). These compounds share a peptidolipid core as found in the GPLs of *M. avium*, but differ in the location and nature of the sugar residues. They do not contain 6-deoxytalose or its derivatives. Moreover, the oligosaccharide portion is linked to the alaninol residue instead of the *allo*-threonine. *M. xenopi* contained GPL of a unique structure (a "non-C-mycoside" GPL) in which the core consisted of a different lipopeptide, fatty acyl-NH-L-Ser-L-Ser-L-Phe-D-*allo*-Thr-OCH_3 (65, 66). The oligosaccharide hapten is linked glycosidically to the *allo*-Thr-OCH_3 residue and contained one or two additional fatty acyl substituents. The remaining 3-*O*-CH_3-6dTal*p* was glycosidically linked to the distal serine residue.

WAXES, ACYLATED TREHALOSES, SULFOLIPIDS Several slowly growing mycobacteria contain an array of waxes, generally long-chain diols [phthiocerols A and B, phthiodiolone, phthiotriol; phthiocerol A is a mixture of 3-methoxy-4-methyl-do-(and tetra)-triacontane-9,11-diols] in which mycocerosic acids are esterified to both hydroxyl groups (see Table 1). Related to this family of waxes are the phenolphthiocerols, which form the basic core of the phenolic glycolipids. Three other families of trehalose-based lipids, cord factor (trehalose 6,6′-dimycolate), the simpler acylated trehaloses [containing a combination of saturated straight-chain C_{16}-C_{19}, C_{21}-C_{25} mycocerosate, C_{24}-C_{28}

mycolipanolic, and C_{25}-C_{27} mycolipenic fatty acids: for example, 2, 3-di-*O*-acylated trehalose isolated from *M. tuberculosis* (67)], and the sulfolipids (trehalose 2′-sulfate acylated with hydroxyphthioceranic, phthioceranic, and saturated straight-chain fatty acids) have been implicated in the pathogenesis of tuberculosis. The structures and biological activities of these have been reviewed (68).

GLYCEROPHOSPHOLIPIDS Although the cell envelope of mycobacteria has a high content of conventional glycerophospholipids, these were assumed to be the components of the plasma membrane. Recent separation of *M. chelonae* cell walls from plasma membranes, with less than 1% contamination by the latter (19), allowed the analysis of pure cell walls, which unexpectedly contained about two short-chain (C_{16-18}) fatty acid residues per bound mycolic acid residue. Some of these short-chain acids were present in PIMs (EY Rosenberg, H Nikaido, unpublished results). Interestingly, earlier studies reported the presence of glycerophospholipids, especially PIM, in crude cell wall fractions of *M. phlei* (69) and *M. tuberculosis* (70), although these reports were greeted with skepticism because of the lack of evidence for the purity of the "cell wall" fractions. Now that we know that glycerophospholipids constitute a fraction of extractable lipids at least in *M. chelonae* cell wall, we need to expand similar quantitative analysis to other mycobacterial species.

OUTER LAYERS AND CAPSULES Some of the extractable lipids may exist outside the cell wall proper, as is discussed below. The case has been made that in *M. tuberculosis* isolates, the exocellular and surface-exposed materials are mostly polysaccharides, specifically D-glucans, D-arabino-D-mannans, and D-mannans (71).

Cell Wall Proteins

The purified cell wall of *M. chelonae* contained 30-kDa major protein (19). Similarly, *M. leprae* cell wall fraction was enriched in a 35-kDa major protein (72). The functions of these proteins are not known. Trias et al (73) isolated a pore-forming protein, porin, from the cell wall of *M. chelonae* by following its channel-forming function. The function of this 65-kDa minor protein is discussed below.

Physical Organization of Cell Wall Lipids

Knowledge of the chemistry of mycobacterial lipids unfortunately does not allow us to understand the function of the cell wall as the permeability barrier. For this purpose, we need to know the physical organization of the lipids. More than 10 years ago, Minnikin proposed a model, in which mycolic acid chains

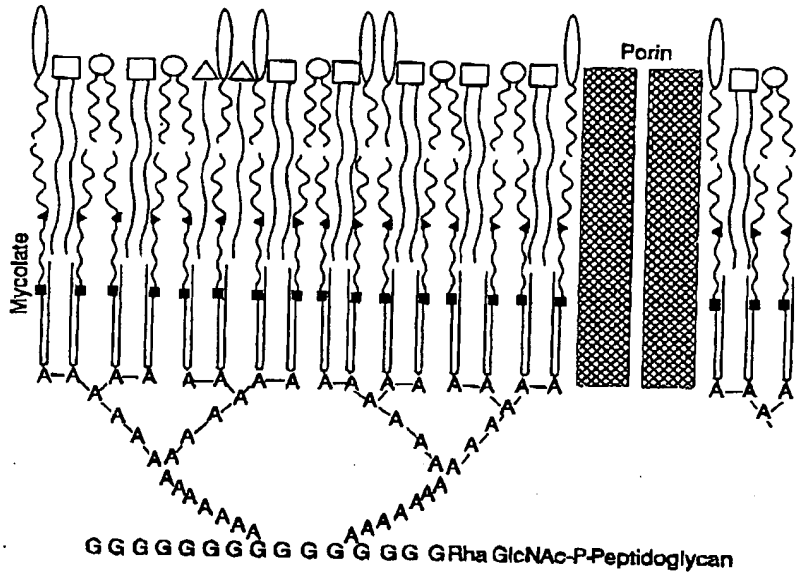


Figure 6 A model of the mycobacterial cell wall. *cis*- or *trans*-Double bond, or *cis*- or *trans*-cyclopropane group (solid squares); *cis*-double bond, *cis*-cyclopropane group, or oxygen-containing groups (solid triangles). The lipids composing the outer leaflet differ from species to species. Those containing short-chain (C_{16-18}) fatty acids include glycerophospholipids and GPL. Those containing intermediate chain-length hydrocarbons include PGL, phthiocerol dimycocerosate, and others.

are packed side by side in a direction perpendicular to the plane of the cell surface (5). It was also proposed that this mycolic acid–containing inner leaflet is covered by an outer leaflet composed of extractable lipids, the whole structure thus producing an asymmetric lipid bilayer (Figure 6).

This model has received support from several recent studies. X-ray diffraction study of purified *M. chelonae* cell wall showed that a large part of the hydrocarbon chains in the cell wall are tightly packed in a parallel array in a direction perpendicular to cell surface (19). An important feature of the X-ray data was that a large fraction of the lipid was in a nearly crystalline arrangement of presumably very low fluidity, whereas the rest showed the diffuse diffraction expected of fluid domains. The coexistence of domains of different fluidities is expected from our consideration of the mycolic acid structure. Thus the section closest to the carboxyl group contains few, if any, structures capable of producing "kinks" that increase fluidity: The α-branch is always without any double bond or cyclopropane group, and the proximal portion of the

meromycolate chain also favors crystalline arrangement because the first double bond or cyclopropane group (solid squares in Figure 6) is often *trans*, which does not introduce kinks (42). [For example, the most abundant mycolic acid species in *M. smegmatis*, α-smegmamycolic acid, contains a *trans* double bond at this position (74).] The disorder increases as one approaches the outer surface, because the second double bond or cyclopropane group (solid triangles in Figure 6) has the *cis* configuration, and at the corresponding position mycolate often contains oxygen-containing substituents that would strongly disrupt the tight lateral packing (Figure 3). The outer leaflet, containing the common glycerophospholipids (see above), should have the usual high fluidity. This large change in fluidity across the thickness of the membrane is reminiscent of the Gram-negative outer membrane, which is composed of a low-fluidity lipopolysaccharide outer leaflet and a high-fluidity inner leaflet (75). The gradient of fluidity in mycobacterial cell wall seems to have an opposite orientation, with the external regions more fluid than the internal segment. This arrangement explains the observation that mycobacterial permeability barrier can be disorganized by adding surfactants from the outside (76), whereas the Gram-negative outer membrane is highly resistant to such treatment (75).

The bilayer model also fits with the electron-microscopic observations discussed already. First, there is a freeze-fracture plane within the cell wall, a finding that is consistent with the presence of bilayer. Second, the bilayer model predicts that the hydrophobic center of the cell wall, consisting of hydrocarbon chains of 70–80 carbon atoms (C_{60} meromycolate chain plus C_{16-18} lipid), would have about twice the thickness of such a layer in the plasma membrane (C_{16-18}, twice). Indeed the transparent layer of the cell wall was twice as thick as that in the plasma membrane, as described above (Figure 1).

Are there enough bound mycolic acids to cover the cell surface as a leaflet, as predicted by the model? Quantitative analytical data on *M. bovis* strain (BCG) (77) indeed suggest that this is the case (19). Finally, the model requires that the cell wall contains enough extractable lipids as components of the outer leaflet. Furthermore, because the α-branch and the meromycolate branch of mycolic acid differ in length (usually by 25 carbons or more), we need lipids containing the short-chain fatty acids with 14–18 carbons, as well as those containing fatty acid residues of about 30–40 residues. Quantitative analysis of fatty acids in *M. chelonae* cell wall showed that it contained not only glycerophospholipids and GPL but also substantial amounts of lipids containing intermediate chain-length fatty acids, although the identity of these lipids is not yet clear (EY Rosenberg, H Nikaido, unpublished results).

The tendency of mycolic acid hydrocarbons to produce tight, parallel arrays was also shown by the pioneering study of Durand et al (78). These authors

demonstrated, by monolayer studies, that a cord factor containing natural, C_{80} di-*cis*-unsaturated mycolic acid residues formed a dense, presumably paracrystalline, packing at room temperature, a result indicating that the interaction between long hydrocarbon chains is strong enough to overcome the disorganizing effect of *cis* double bonds. In this study, cord factors containing corynemycolate (C_{32}) of *R* or natural configuration around the β-carbon had a much higher enthalpy of melting in comparison with a similar compound with the unnatural *S* configuration, or an analog containing β-deoxy corynemycolic acids. This finding suggests that the β-OH moiety stabilizes the mycolic acid monolayer (the inner leaflet in the bilayer of Figure 6) by intermolecular or intramolecular hydrogen bonding. This is reminiscent of the model that in the outer membrane of Gram-negative bacteria, the outer leaflet, which acts as the main permeability barrier, is stabilized by hydrogen bonding involving the β-oxygen atom of β-hydroxymyristate residues of the component lipid, lipopolysaccharide (79).

We believe that extractable lipids usually occur in the outer leaflet of the bilayer. Some lipids, however, may form an independent aggregate outside the bilayer, especially when overproduced. For example, PGLs of *M. leprae* are quite apolar and are likely to form disordered oil droplets, as do such apolar lipids as triglycerides. Indeed, PGL-I is known to occur as loosely cell-associated lipid droplets on the surface of *M. leprae* (80).

Amphiphilic lipids associate to produce organized structures in water. When the cross-section of head group is equal to that of the hydrophobic tail, the lipid tends to form bilayers, but when the cross-section of head group is larger than that of the hydrocarbon tail, the lipid forms micelles or fibrillar structures (as extension of micelles in one direction) (81). Among the free lipids, GPLs appear to fall into this category. They contain a large head group, containing 3–6 sugar units and 4 amino acids, and yet their hydrocarbon moiety consists of only one fatty acid chain (except in *M. xenopi* GPL). Indeed, *M. lepraemurium* produces fibrillar material on its surface, and this material appears to consist of GPLs (82). A similar observation was made with *M. avium* (83).

In contrast to PGL and GPL, trehalose dimycolate (cord factors), LOSs, the trehalose-containing sulfolipids, as well as more conventional glycerophospholipids such as PIMs, are ideally shaped to form bilayers, and thus are unlikely to exist outside the cell wall bilayer.

The bilayer model also fits with the unusual structure of AG. Thus there are many (usually around 12–13) Araf residues between the mycolate residues and the central galactan chain (Figure 2). The presence of these many Ara residues and the exceptional flexibility of Araf-(1→5)-Araf linkages are expected to facilitate the lateral movement of mycolate hydrocarbons, helping their tight packing into a rather rigid structure. Such packing would otherwise be difficult,

because about 16 mycolic acid residues are covalently connected to one AG molecule.

LAM and lipomannan were thought to be anchored to the plasma membrane through their PIM structures. However, now that PIM is known to exist in the cell wall bilayer, it seems equally possible that they are anchored to the outer leaflet of the cell wall. In this arrangement, the hydrophilic polysaccharide chains need not be assumed to penetrate the hydrocarbon interior of the cell wall.

MYCOBACTERIAL CELL WALL AS A PERMEATION BARRIER

Small solutes are expected to traverse the mycobacterial cell wall either through the porin channel (73) or through the lipid bilayer region, just as they traverse the Gram-negative outer membrane by using either of these mechanisms (75). Since hydrophilic solutes cannot traverse lipid bilayers, they are predicted to utilize the porin pathways in the mycobacterial cell wall as well. In contrast, lipophilic solutes are not the favored solutes for passage through porin channels (75) and thus are likely to diffuse mainly through lipid bilayers.

Permeability to Hydrophilic Solutes

The permeability of the *M. chelonae* cell wall to hydrophilic solutes cephalosporins, was experimentally determined (84) with a method introduced for measurement of outer membrane permeability in Gram-negative bacteria (85). The rate of hydrolysis of cephalosporins by intact mycobacterial cells was measured, and the cell wall permeability was calculated by assuming that drug molecules first diffuse through the cell wall (following Fick's first law of diffusion) and then are hydrolyzed by periplasmic β-lactamase (following Michaelis-Menten kinetics). The cell wall permeability measured was indeed very low: about 3 orders of magnitude lower than that of *Escherichia coli* outer membrane and 10 times lower than the permeability of the notoriously impermeable *Pseudomonas aeruginosa* outer membrane. Permeation rates had low temperature coefficients and did not increase when more lipophilic cephalosporins were used (84); these data suggest that the permeation occurred mainly through aqueous channels. Permeability to hydrophilic nutrient molecules such as glycerol and glucose was also very low (84).

When the presence of a porin was discovered in *M. chelonae* and its properties were determined (73), it became clear that the low hydrophilic permeability of mycobacteria is due to two factors. First, *M. chelonae* porin is a minor protein of the cell wall, unlike enterobacterial porins (73). Second, *M. chelonae* porin produces permeability far lower than that produced by an equal weight of *E. coli* porin (73). The presence of a pore-forming protein with

similar properties was shown in *M. smegmatis* (86), and similar porins are probably distributed widely among mycobacteria.

The mycobacterial cell wall thus shows an unusually low degree of permeability to hydrophilic solutes. However, there are significant differences among various species. Recent studies showed that in *M. smegmatis* (86) and *M. tuberculosis* H37Ra (EY Rosenberg, H Nikaido, unpublished results), the cell wall permeability to β-lactams is about an order of magnitude higher than in *M. chelonae*. These results may be compared with the presumptive indicators of hydrophilic permeability shown in Table 1. Higher sensitivity to a small, hydrophilic inhibitor hydroxylamine indeed suggests that *M. tuberculosis* and *M. smegmatis* cell walls are more permeable than *M. chelonae* cell wall, as expected. It is also consistent with the earlier observation that nutrients are accumulated more rapidly by *M. smegmatis* (reviewed in 87) and by *M. tuberculosis* (88) than by *M. chelonae*. Finally, if the sensitivity to *p*-nitrobenzoate indeed gives some indication of hydrophilic permeability, we can also predict that most of the slow growers listed in Table 1 should have lower hydrophilic permeability than *M. tuberculosis*. These species are indeed more resistant to a small, hydrophilic antimycobacterial agent, ethambutol, than is *M. tuberculosis* (15a).

Permeability to Hydrophobic Solutes

A lipid bilayer is ordinarily highly permeable to lipophilic solutes. However, its permeability is inversely correlated with its fluidity (42). Fluidity decreases when the membrane lipid contains longer hydrocarbon chains with fewer *cis*-double bonds or *cis*-cyclopropane groups. The innermost part of mycobacterial cell wall is extreme in this regard, and presumably has very low fluidity. Furthermore, lipids of the type in which more than two fatty acid chains are connected to a single head group appear to reduce the permeability further (89); mycolyl-AG is an example of this type of lipid.

These principles were experimentally verified with the outer membrane of Gram-negative bacteria. There the outer leaflet consists of lipopolysaccharide, which contains 6–7 saturated fatty acid residues, all connected to a single head group (75). The permeability of the outer membrane bilayer, most probably limited by the lipopolysaccharide leaflet, was indeed up to 100-fold lower than the permeability of bilayers composed of the usual glycerophospholipids (90). Since mycobacterial cell wall is more extreme in its structure, we can expect even lower permeability through its lipid matrix. However, this pathway cannot be neglected, because it may make a more significant contribution to the permeation of hydrophobic solutes than the porin pathway, which is extremely inefficient as described above. Very lipophilic solutes cross the asymmetric bilayer of the Gram-negative outer membrane at rates similar to those with which β-lactams traverse *E. coli* porin channels (90). Since the permeability

through the porin channels is about 100–1000-fold lower in the mycobacterial cell wall, the lipid bilayer pathway may remain more important for such solutes than the porin pathway, even if this bilayer is less permeable, say by a factor of 10, than that of the Gram-negative outer membrane.

The antibacterial agents of the relatively lipophilic classes, such as rifamycins, tetracyclines, macrolides, and fluoroquinolones, may thus utilize the lipid bilayer pathway in traversing the mycobacterial cell wall. If so, one can predict that, within each class, the more lipophilic derivatives would be more active against mycobacteria. This is indeed the case. For example, among fluoroquinolones, the more hydrophobic sparfloxacin is more active than the reference fluoroquinolones against many mycobacteria (91). Ciprofloxacin, when made more hydrophobic by the addition of alkyl substituents, becomes more active against *M. tuberculosis* and *M. avium* (92). With *M. leprae*, a good positive correlation was seen between the lipophilicity of fluoroquinolone and its efficacy (93; the data are analyzed in 87). Similar examples exist for other classes of agents: tetracyclines (94–96), macrolides (97–99), and rifamycins (100). In *M. avium*, which appears to have very low hydrophilic permeability, the efficacy of a small, hydrophilic agent isoniazid was improved by converting it into a hydrophobic compound, by the addition of palmitoyl substituent (101). Recently, the penetration rate of norfloxacin, a fluoroquinolone, into *M. tuberculosis* cells was shown to increase more than six times when the temperature was increased by 10°C (T Kocagoz, HF Chambers, personal communication), and this high temperature coefficient also suggests the predominant role of the lipid bilayer pathway in penetration.

We can also compare the activity of one single agent against various mycobacterial species. Table 1 shows that several fast growers, especially *M. chelonae* and *M. fortuitum*, are highly resistant to lipophilic inhibitors such as dyes and detergents. Such differences in susceptibility presumbaly reflect differences in cell wall permeability, which are also likely to affect the susceptibility of various species to lipophilic antibiotics. For example, rifampicin is active against almost all of the clinically relevant species of mycobacteria, except *M. chelonae* and *M. fortuitum* (and *M. avium* complex among the slow growers) (102). Available data indeed allow us to propose a structural explanation of such differences in permeability. Thus in resistant species (*M. chelonae*, and *M. fortuitum*, *M. smegmatis*, and *M. phlei* as well as a relatively resistant slow grower, *M. terrae*), substantial fractions (41-75%) of α-mycolates, which constitute a major fraction of mycolates in most organisms, contain *trans* double bonds at the proximal (inner) position (Table 1). These *trans* double bonds will decrease the fluidity, and thus permeability, of the innermost part of the bilayer (Figure 6), the part that would act as the rate-limiting permeability barrier for lipophilic solutes. In contrast, in the more susceptible species (*M. thermoresistible*, *M. vaccae*, and *M. aurum* among fast growers as

well as most of the slow growers), fluidity and permeability of the innermost part are presumably much higher, because most of their α-mycolates contain fluidity-increasing *cis* double bonds or *cis* cyclopropane groups at the corresponding position (Table 1). Although the α-mycolates of *M. avium* group appear to contain some *trans* cyclopropane structures at the proximal position, their high reistance to lipophilic agents cannot be explained by this observation alone.

Cell Wall Barrier is a Necessary, but not a Sufficient, Factor for Resistance

Although mycobacterial cell wall is a formidable permeation barrier, production of clinically significant levels of resistance usually requires the participation of an additional resistance mechanism, such as the enzymatic inactivation or the active efflux of the agents. This is because the surface-to-volume ratio is extremely high in small bacterial cells, and thus half-equilibration across the cell wall takes place in several minutes for β-lactams, even though the permeability coefficients of the mycobacterial cell wall are exceedingly low (2). The nature of these additional resistance mechanisms for mycobacteria is discussed in Ref. 2.

MYCOBACTERIAL CELL WALL: INTERACTION WITH HOST COMPONENTS

The cell wall and its associated structures are the outermost components of a bacterial cell, and as such they play crucial roles in the interaction of pathogenic species with their host. Below we discuss some of these "cell surface" functions of the components of mycobacterial cell wall.

LAM exhibits a wide spectrum of immunoregulatory functions, but the biological implication of the in vitro data is not always clear. Earlier data using *M. leprae* LAM and AraLAM from a rapidly growing *Mycobacterium* sp. were interpreted as a suggestion that LAM suppresses immune responses, thus contributing to pathogenesis of tuberculosis and leprosy. These data include LAM-induced abrogation of T-cell activation (104), inhibition of γ-interferon-mediated activation of murine macrophages (105), scavenging of potentially cytotoxic oxygen free-radicals (106), and inhibition of protein kinase C activity (106). Although AraLAM evoked a large array of cytokines associated with macrophages, such as α-tumor necrosis factor (TNF) (107), granulocyte macrophage-colony stimulating factor, and interleukins-1a, 1b, 6, and 10 (108), this was frequently interpreted as a contributor to the disease processes: for example, the production of fever, weight loss, and tissue necrosis was emphasized in the case of α-TNF. More recently, however, ManLAM, which is present in strains of *M. tuberculosis*, was found to be much less potent in

evoking α-TNF, in contrast to AraLAM found in nonvirulent species (107). Similarly, AraLAM, but not ManLAM, was found to activate the early response genes (including c-*fos* and the genes for α-TNF) in macrophages (109). Additionally, ManLAM could stimulate phagocytosis by interacting with the Man receptor (110). These results now suggest that *M. tuberculosis* strains become phagocytized efficiently but survive within the host macrophages because their ManLAM does not activate these phagocytes.

The glycolipids of PGL class are located at cell surface, and may in some cases exist even outside the bilayer structure of the cell wall proper, as described above. They have been implicated in pathogenesis. For example, PGL is thought to contribute to the intracellular survival of *M. leprae* within macrophages of individuals with lepromatous leprosy through its ability to scavenge oxygen radicals (61). The variable oligosaccharide constituents of these glycolipid antigens are usually of sufficient antigenicity as to evoke corresponding specific antibodies and thereby allow serodiagnosis of individual mycobacterioses (8) and leprosy (61). The triglycosyl phenolphthiocerol dimycocerosate of *M. tuberculosis*, PGL-Tb1 (111), is apparently not present in virulent strains of *M. tuberculosis* and therefore, unlike PGL-I from *M. leprae*, is not useful in the serodiagnosis of tuberculosis.

GPLs are also clearly present on the bacterial cell surface, as anti-GPL antibodies were repeatedly shown to react with intact cells. It has been pointed out above that some GPLs could exist as micelles or fibrils outside the cell wall bilayer proper. Species that produce large amounts of GPLs tend to produce smooth colonies, presumably because the surface of their cells is covered by hydrophilic carbohydrate moieties of GPL. In contrast, typical strains of *M. tuberculosis* are devoid of GPL, typical LOS, or PGL, and indeed produce colonies with extremely hydrophobic, rough surfaces (15). Carbohydrate layers on cell surfaces usually contribute to virulence by preventing nonspecific phagocytosis, but it is not clear whether this is also the case with mycobacteria, which survive within macrophages. Indeed both smooth, GPL-containing forms and rough, GPL-deficient forms of *M. avium* are isolated from patients (112). GPL was also often suggested to protect mycobacterial cells within the phagolysosomes, but GPL-deficient rough mutants of *M. avium* were as resistant in this environment as the smooth parent strain (113).

BIOSYNTHESIS AND ASSEMBLY

Arabinogalactan (AG)

The structure of the AG linker [α-L-Rha*p*-(1→3)- D-GlcNAc (1→P)] is strikingly similar to that of the linker involved in the attachment of teichoic acid to peptidoglycan, *N*-ManNAc-*N*-GlcNAc-1-P (114). The linkage unit plays a

key role in the initiation of new teichoic acid chains (115). Thus the biosynthesis begins by the transfer of GlcNAc-1-P moiety from UDP-GlcNAc onto a polyisoprenoid lipid carrier in a reaction that is inhibited strongly by tunicamycin (116, 117). This reaction is then followed by the assembly, still on the polyisoprenoid carrier, of the rest of the linker and the teichoic acid proper. Since similar linkage units are also involved in cell wall attachment of a polysaccharide in *Micrococcus luteus* (118), and of teichoic acids in actinomycetes (119), we may assume that AG is also synthesized in a similar way. If so, the first reaction will be the transfer of GlcNAc-1-P moiety from UDP-GlcNAc to a polyprenol carrier, followed by the transfer of L-rhamnosyl moiety (presumably from dTDP-Rha). This is presumably followed by the transfer of Gal*f* units. Although the donor of Gal*f* has not been identified in mycobacteria, UDP-Gal*f* is the donor of Gal*f* in polysaccharide synthesis in *Penicillium charlesii* (120). Ara*f* is also likely to be added to the growing polysaccharide while it is still linked to the carrier. Mycobacterial cell extracts catalyze the transfer of Ara*f* from Ara*f*-P-decaprenol to AG and LAM (K Mikusova, PJ Brennan, unpublished results); the nucleotide-linked form of Ara*f*, however, is not known.

The sites of action of several antituberculosis drugs are probably located in this pathway. Ethambutol at 3.0 μg/ml inhibited the transfer of label from [^{14}C]Glc into the D-Ara residue of AG in whole cells of a drug-susceptible strain of *M. smegmatis* (121), inhibition that began almost immediately upon exposure of the cells to the drug. This dramatic decline in incorporation of label in growing *M. smegmatis* applies to the arabinan moiety of both AG and LAM (K Mikusova, PJ Brennan, unpublished results). Thus the primary mode of action of ethambutol appears to be the inhibition of synthesis of the arabinan of both AG and LAM.

Mycolic Acids

Early studies on the biosynthesis of mycolates showed that in *Corynebacterium diphtheriae*, C_{32} mycolic acids were formed by Claisen-type condensation and reduction of C_{16} fatty acids (122), and similar condensations were established for the mycolic acids of *Nocardia asteroides* and *M. smegmatis* (123). The proposed pathway for biogenesis of mycolates (Figure 7) involves four stages (124): (*a*) synthesis of C_{24}-C_{26} straight-chain saturated fatty acids to provide C-1 and C-2 atoms and the α-alkyl chain; (*b*) synthesis of C_{40}-C_{60} acids (meromycolic acids) to provide the main carbon backbone; (*c*) modification of this backbone to introduce other functional groups—one series of C_{24} to C_{32} acids are produced by a Δ-5-desaturase enzyme acting on a C_{24} acid, followed by elongation, implying that at least some of the modifications are introduced during growth of the meromycolate chains; and (*d*) the final condensation step to produce mycolic acid.

There is evidence that the point of divergence from the biosynthesis of conventional fatty acids is at the C_{24} level, with a ω-19 acid, 24:1 *cis*-5, being elongated (124a). ω-19 Acids have been detected at low concentrations in lipid extracts of mycobacteria (125), and these acids correspond structurally to the methyl terminus of the meromycolate chain. Incubation of mycobacterial cell walls with labeled acetate and extraction and oxidation of resulting mycolates to fragment the meromycolate chain at its double bonds demonstrated that the methyl terminus and the next 18 carbon atoms were virtually unlabeled (126), indicating an endogenous ω-19 precursor. Also, when wall material was extracted with hexane to remove endogenous fatty acids, synthetic 24:1 *cis*-5 significantly stimulated the incorporation of labeled acetate into mycolic acids, while a range of other acids, even some 24-carbon acids, had no effect.

The early steps in the biosynthesis of mycolic acids have not been defined (124a). Direct incorporation of appropriate labeled fatty acids such as 24:1 *cis*-5 into mycolic acids is yet to be shown. The carrier molecule must be identified. Coenzyme A (CoA) is the usual carrier, but it does not function in these cell-free systems (126). Further, it is not known how mycobacteria synthesize 24:1 *cis*-5; a 24:0 desaturase has been characterized, but its product is the ubiquitous 24:1 *cis*-15 acid, nervonic acid (127). The starting 24- and 26-carbon fatty acids are probably synthesized by fatty acid synthases (FASs) and elongases, specifically the FAS-I and -II complexes (128). In *M. tuberculosis*, these functions are linked in a multifunctional enzyme system that appears to be a de novo synthase joined to an elongase that elongates C_{16} to C_{24} or C_{26} (129). Further elongation of the C_{24} fatty acids to meromycolic acids (C_{50}-C_{56}) has been demonstrated (124, 130), but it is not clear which of these FAS/elongases are used.

α-Mycolate, which contains double bonds or cyclopropane rings as its only functional groups, is not a precursor of the more complex oxygenated mycolates. The dissociation of their biosynthesis was shown in *M. aurum*, in which radioactive label appeared in ketomycolate before α-mycolate (131). Thus, any common intermediate must be at an early stage in biosynthesis. For this, 24:1 *cis*-5 is a good candidate; judging from the structure of oxygenated mycolates, it would be expected to be oxidized and methylated to a 5-keto, 6-methyl-24-carbon precursor. Mutants and isolates impaired in mycolic acid synthesis are known, e.g. strains of actinomycetes (132), and one mutant of *M. smegmatis* that synthesizes only the unbranched meromycolates (133). These should be useful in unraveling this complex pathway.

The unique mycolyl phospholipid (Myc-PL), 6-*O*-mycolyl-β-D-mannopyranosyl-phosphooctahydroheptaprenol, has the attribute of a mycolyl carrier and may play a role in the terminal mycolyl group transfer to cell wall components. The Myc-PL is probably the product of a Claisen-type condensation reaction and contains an acyl carrier group that is equivalent to the proposed R_1 in

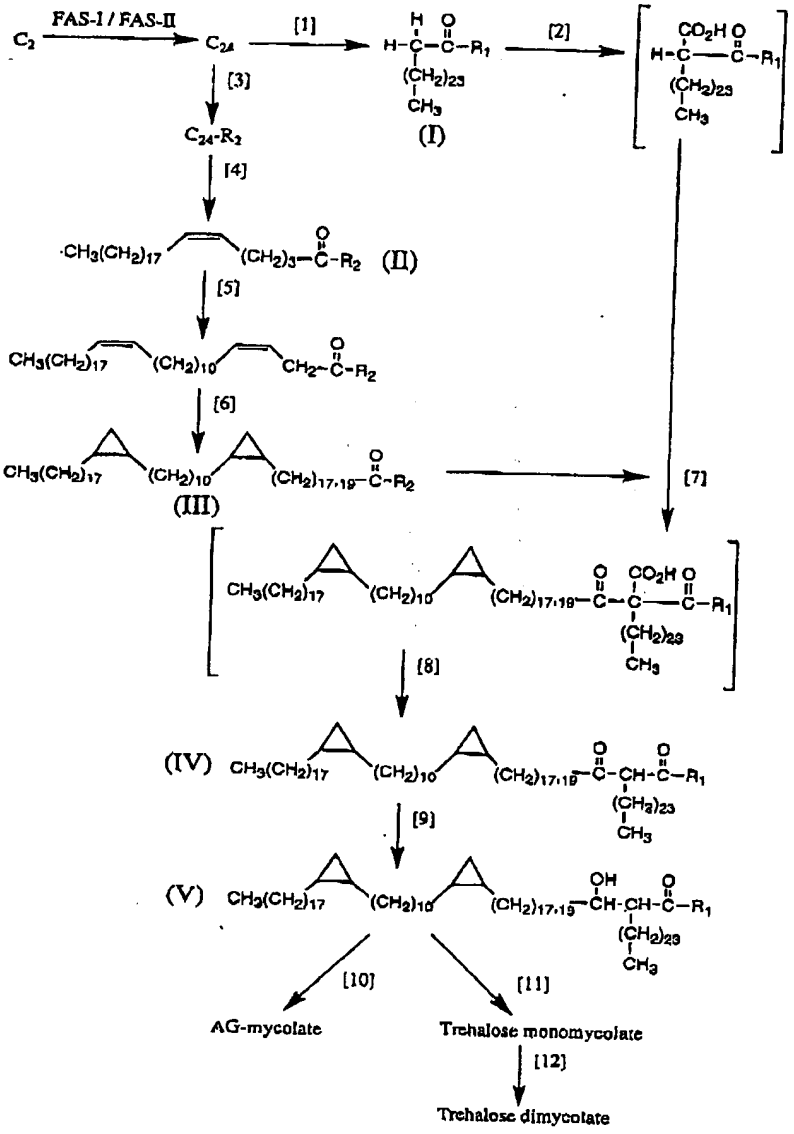

FAS-I / FAS-II
C2
C24
[1]
[2]
[3]
C24-R2
[4]
(I)
(II)
[5]
[6]
(III)
[7]
[8]
(IV)
[9]
(V)
[10]
[11]
AG-mycolate
Trehalose monomycolate
[12]
Trehalose dimycolate

Figure 7. The other acyl carrier group R_2 could be either CoA, acyl carrier protein (ACP), or membrane-bound protein. Myc-PL may be the direct precursor of trehalose monomycolate (TM), or it may transfer the mycolate residue to trehalose (partial reaction [11] in Figure 6). Presumably, the fully formed mycolic acid is transferred to AG to form the mycolate-AG complex either from Myc-PL (partial reaction [10]) or TM (not shown). The Claisen-type condensation of the C_{26} fatty acid with the meromycolic acid to yield the ketomycolyl-R_1 intermediate (partial reactions [7, 8] in Figure 7) has not been demonstrated in a cell-free system. However, the synthesis of the short-chain C_{32}-C_{36} corynemycolic acids has been reported in vitro using *C. diphtheriae* (134) and *C. matruchotii* (135). These products were reported to appear in the form of TM. These esters were thought to be carriers of the mycolic acid to the mycobacterial cell wall, where it is transferred to the nonreducing terminal D-arabinose residue of AG via an unknown transacylation reaction.

The strongest evidence that normal mycolic acid metabolism is crucial to the survival of *M. tuberculosis* comes from work on the action of two well-known antituberculosis drugs, isoniazid and ethionamide, which apparently inhibit primarily mycolic acid synthesis (136). Identification of the *inhA* resistance gene seems to implicate the elongation stages of mycolate synthesis in isoniazid action rather than the condensation step and beyond. Ethionamide and isoniazid share similar inhibitory properties, and the missense mutations within the *inhA* gene confer resistance to both drugs (137). All of the features of the gene (homology to *envM*, NAD/NADH binding, downstream from a β-ketoacyl ACP reductase; 137; WR Jacobs, personal communication) point to an enoyl ACP reductase and therefore involvement in elongation events. Long before the genetic evidence, biochemical data suggested that isoniazid specifically inhibits the insertion of a Δ-5 double bond into a C_{24} fatty acid (138).

Extractable Lipids

The biosynthesis and genetics of only some of the glycolipids have been examined. Biosynthetic pathways for assembling the GPLs of *M. avium* had been proposed earlier (8), but these schemes remain to be confirmed. Recent

Figure 7 Proposed anabolic pathway of mycolic acids in *M. tuberculosis* H37Ra. This is an updated version of a previously proposed pathway (46). The key products are (I) hexacosanoate-R_1, (II) Δ-5-tetracosanoate-R_2, (III) $C_{52,54}$ meromycolate-R_2, (IV) oxomycolate-R_1, and (V) mycolate-R_2. Carrier group R_1 is believed to be the β-D-mannopyranosyl-monophosphoryl-polyisoprenol, and R_2 may be either CoA or ACP. The reactions are identified as follows: [1] elongation and introduction of carrier group R_1, [2] carboxylation, [3] introduction of carrier group R_2, [4] Δ-5-desaturation, [5] elongation and Δ-3-desaturation, [6] introduction of cyclopropane rings and elongation, [7] Claisen-type condensation, [8] decarboxylation, [9] reduction, [10] mycolate transfer to AG, [11] mycolate transfer to trehalose, and [12] trehalose mycolyltransferase. (Courtesy of Dr. G.S. Besra.)

publications (139–141) presented a novel approach in this area. The genes that confer serovar specificity to *M. avium* serovar 2, designated the *ser2* cluster, were cloned, expressed in *M. smegmatis*, and shown to encode the determinants necessary to synthesize the haptenic oligosaccharide segment of the serovar 2–specific GPL, i.e. the distal disaccharide 2,3-di-*O*-methyl-α-L-fucopyranosyl-(1→3)-α-L-rhamnopyranose. Expression of the *ser2* genes in *M. smegmatis* produced a recombinant serovar 2–specific GPL, because the nonspecific GPL of *M. smegmatis*, singly glycosylated at the *allo*threonine residue with a 6-deoxytalose, served as a precursor for further, serovar 2–directed glycosylation. Recently, transposon mutagenesis defined four essential loci within the *ser2* gene cluster, encoding at least the rhamnosyltransferase, the fucosyltransferase, and the methyltransferases required to methylate the fucose (142). Further, isolation of the truncated versions of the hapten induced by the transposon insertions provides genetic evidence that the GPLs of *M. avium* serovar 2 are synthesized by an initial transfer of the rhamnose unit to the 6-deoxytalose attached to the peptide core followed by fucose and finally *O*-methylation of the fucosyl unit.

There is also some limited information on the biosynthesis of PGLs and their components (143). Early work demonstrated that the carbon atoms in the methyl-branched structures in mycocerosic acids are derived from propionate (144, 145). So also are those in phthiocerol (146). The phenolphthiocerols probably come from *p*-hydroxybenzoate, which are then elongated to yield the long-chain diol (146). The methoxyl residue in phenolphthiocerol presumably comes from methionine, by analogy with its known source in phthiocerol. Recently, *M. microti* was shown to contain two lipids, which became labeled when the cells were grown in the presence of [2-^{14}C]propionate (143). These were identified as phenolphthiocerol dimycocerosate and phenolphthiodiolone dimycocerosate, the aglycosyl derivatives of mycoside B, the phenolic glycolipid produced by *M. microti* and *M. bovis*. Cell-free extracts of the organism were able to glycosylate the lipids to form mycoside B in vitro.

Some of the enzymes for biosynthesis of the mycocerosates have been purified, and their genes cloned and sequenced (147, 148). Like some of the fatty acid synthases (133), mycocerosic acid synthase (MAS) is a multifunctional enzyme with domains that are involved in fatty acid elongation (149). Its unusual features are its substrate specificity for methylmalonyl-CoA, an acyl carrier protein–like domain that is usually associated with aggregated enzyme systems and a product mycocerosate (2,4,6,8-tetramethyloctacosanoate) that remains bound to the enzyme. The role of the acyl carrier protein–like domain may be in binding the product, and there apparently is no thioesterase domain to release the product, perhaps to prevent uncontrolled acylation of lipids with mycocerosates. The methylmalonyl-CoA is generated by the same

acyl-CoA carboxylase that generates malonyl-CoA for straight-chain fatty acid elongation in *M. tuberculosis* (150).

OUTLOOK AND CHALLENGES

Many areas remain to be explored for our better understanding of the structure and functions of the mycobacterial cell wall. In terms of structure, our knowledge of the lipids that presumably form the outer leaflet of the bilayer structure is still inadequate, and we need to define the precise arrangement of these lipids. Further, a major challenge is to understand the mechanism of assembly of this extremely complex structure, which appears to have a rather rigid interior. In terms of function, we are only beginning to get a glimpse of the possible functions of some lipid species. Mycobacterial cells must synthesize so many lipid species of unusual structures because each species performs important functions in their interactions with the nonliving or living environment. Our knowledge on the latter process, that is the role of cell wall components in pathogenesis, is unsatisfactory in spite of great efforts over many years. Perhaps a major reason for the lack of success is that we know so little about pathogenesis. Much work has been done to study the roles of cell wall components in protecting mycobacteria against oxygen radicals and in preventing the phagolysosome fusion, the two mechanisms long believed to be crucial for the intraphagocytic survival of mycobacteria. Yet recently it was reported that the major macrophage factor responsible for killing of mycobacteria is reactive nitrogen intermediates, rather than oxygen radicals (151), and that *M. tuberculosis* apparently multiplies within macrophages by escaping from the fused phagolysosomes rather than by preventing the fusion event (152). In any case, we hope that a better understanding of the functions and biosynthetic pathways of these cell wall components will be achieved, especially because recombinant DNA methods can now be used with mycobacteria (153), and that this may enable us to develop strategies that are more effective in controlling these unusually resistant pathogens.

ACKNOWLEDGMENTS

Studies in our laboratories have been supported by NIH grants AI-09644 and -33702 (to HN) and AI-18357 (to PJB).

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Serial No.: 29/196,311 Filing Date: December 29, 2003

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Tuberculosis (2003) 83, 91–97

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Structure, function, and biogenesis of the cell wall of *Mycobacterium tuberculosis*

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Summary Much of the early structural definition of the cell wall of *Mycobacterium* spp. was initiated in the 1960s and 1970s. There was a long period of inactivity, but more recent developments in NMR and mass spectral analysis and definition of the *M. tuberculosis* genome have resulted in a thorough understanding, not only of the structure of the mycobacterial cell wall and its lipids but also the basic genetics and biosynthesis. Our understanding nowadays of cell-wall architecture amounts to a massive "core" comprised of peptidoglycan covalently attached *via* a linker unit (L-Rha–D-GlcNAc-P) to a linear galactofuran, in turn attached to several strands of a highly branched arabinofuran, in turn attached to mycolic acids. The mycolic acids are oriented perpendicular to the plane of the membrane and provide a truly special lipid barrier responsible for many of the physiological and disease-inducing aspects of *M. tuberculosis*. Intercalated within this lipid environment are the lipids that have intrigued researchers for over five decades: the phthiocerol dimycocerosate, cord factor/dimycolyltrehalose, the sulfolipids, the phosphatidylinositol mannosides, etc. Knowledge of their roles in "signaling" events, in pathogenesis, and in the immune response is now emerging, sometimes piecemeal and sometimes in an organized fashion. Some of the more intriguing observations are those demonstrating that mycolic acids are recognized by CD1-restricted T-cells, that antigen 85, one of the most powerful protective antigens of *M. tuberculosis*, is a mycolyltransferase, and that lipoarabinomannan (LAM), when "capped" with short mannose oligosaccharides, is involved in phagocytosis of *M. tuberculosis*. Definition of the genome of *M. tuberculosis* has greatly aided efforts to define the biosynthetic pathways for all of these exotic molecules: the mycolic acids, the mycocerosates, phthiocerol, LAM, and the polyprenyl phosphates. For example, we know that synthesis of the entire core is initiated on a decaprenyl-P with synthesis of the linker unit, and then there is concomitant extension of the galactan and arabinan chains while this intermediate is transported through the cytoplasmic membrane. The final steps in these events, the attachment of mycolic acids and ligation to peptidoglycan, await definition and will prove to be excellent targets for a new generation of anti-tuberculosis drugs.

Introduction

Much of the early structural definition of the cell wall of *Mycobacterium* spp. was initiated at the University of Osaka, Japan, and the CNRS, France,

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1472-9792/03/$ - see front matter © 2003 Published by Elsevier Science Ltd.
doi:10.1016/S1472-9792(02)00089-6

by Yamamura, Kato, Azuma, Lederer, and colleagues in the 1960s and 1970s. There was a long period of inactivity, but more recent developments in analytical techniques combined with definition of the *M. tuberculosis* genome have resulted in a thorough understanding, not only of the structure of the mycobacterial cell wall and its lipids but also the basic genetics and biosynthesis.

The cell wall is composed of two segments, upper and lower. Beyond the membrane is peptidoglycan (PG) in covalent attachment to arabinogalactan (AG), which in turn is attached to the mycolic acids with their long meromycolate and short α-chains. This is termed the cell wall core—the mycolyl arabinogalactan–peptidoglycan (mAGP) complex. The upper segment is composed of free lipids, some with longer fatty acids complementing the shorter α-chains, and some with shorter fatty acids complementing the longer chains. Interspersed somehow are the cell-wall proteins, the phosphatidylinositol mannosides (PIMs), the phthiocerol-containing lipids, lipomannan (LM), and lipoarabinomannan (LAM). When cell walls are disrupted, for instance extracted with various solvents, the free lipids, proteins, LAM, and PIMs are solubilized, and the mycolic acid–arabinogalactan–peptidoglycan complex remains as the insoluble residue. In simplistic terms, it can be considered that these lipids, proteins, and lipoglycans are the signaling, effector molecules in the disease process, whereas the insoluble core is essential for the viability of the cell and should be addressed in the context of new drug development.

The cell wall core, the mAGP complex

Structure of mAGP

A spate of intensive investigations from 1950 to 1975 allowed the definition of the insoluble cell-wall matrix as a cross-linked PG linked to AG, and esterified at the distal ends by the mycolic acids. Historically, PG is thought to consist of alternating units of N-acetylglucosamine (GlcNAc) and a modified muramic acid (Mur). The tetrapeptide side chains of PG consist of L-alanyl-D-isoglutaminyl-*meso*-diaminopimelyl-D-alanine (L-Ala–D-Glu–A_2pm–D-Ala) with the Glu being further amidated.[1–4] This type of PG is one of the most common found in bacteria.[5] However, mycobacterial PG differs in two ways: some or all of the Mur residues are N-acylated with glycolic acid (MurNGly), and the cross-links include a proportion between two A_2pm residues as well as between A_2pm and D-Ala.[1,2]

It was known, even in the 1950s, that the major cell-wall polysaccharide is a serologically active branched-chain AG with the arabinose (Ara) residues forming the reducing termini. A structural formula, which proved incorrect, was proposed consisting of repeating units of 11–16 sugar residues. There was some uncertainty about the structure of the galactan, i.e., whether α1→4-linked Gal*p* or α1→5-linked Gal*f*.[6] We and others had shown that the polymer is unique not only in its elemental sugars but, unlike most bacterial polysaccharides, it lacks repeating units,[7] comprised instead of a few distinct structural motifs.[6,8] Partial depolymerization of the per-*O*-alkylated AG and analyses of the generated oligomers by GC-MS and FAB-MS established that: (i) the Ara and Gal residues are in the furanose form; (ii) the non-reducing termini of arabinan consists of the structural motif [β-D-Ara*f*-(1→2)–α-D-Ara*f*]$_2$–3,5-α-D-Ara*f*-(1→5)–α-D-Ara*f*; (iii) the majority of the arabinan chains consist of 5-linked α-D-Ara*f* residues with branching introduced by 3,5-α-D-Ara*f*; (iv) the arabinan chains are attached to C-5 of some of the 6-linked Gal*f* residues, and approximately two to three arabinan chains are attached to the galactan core; (v) the galactan regions consist of linear alternating 5- and 6-linked β-D-Gal*f* residues; (vi) the galactan region of AG is linked to the C-6 of some of the MurNGly residues of PG *via* a special diglycosyl-P bridge, α-L-Rha*p*-(1→3)–D-GlcNAc-(1→P);[9] (vii) the mycolic acids are located in clusters of four on the terminal hexaarabinofuranoside, but only two-thirds of these are mycolated.[10] More recently, we obtained oligosaccharide fragments containing up to 26 glycosyl residues from which molecular weights and alkylation patterns were determined by FAB-MS.[11] The extended non-reducing ends of the arabinan were shown to consist of a tricosarabinoside ("23mer"), with three such units attached to the galactan unit. The galactan was also isolated and was found to consist of 23 Gal residues of the repeating linear structure, [β-D-Gal*f*-(1→5)–β-D-Gal*f*-(1→6)]$_n$, devoid of any branching, thereby demonstrating that the points of attachment of the arabinan chains are close to the reducing end of galactan, itself linked to PG *via* the linker disaccharide-P.

Biosynthesis of mAGP

The importance of understanding the biosynthesis of the mycolic acid–peptidoglycan–arabinogalactan complex is more in the context of new drug development against tuberculosis (TB) and less in

defining the bacterial factors responsible for the disease process.

One of the great developments in recent years in the chemical definition of the cell-wall core was the recognition of the diglycosyl-P bridge lying between the linear PG and the linear galactan. We immediately speculated, based on cell-wall biosynthesis in other bacteria, that the entire linkage unit, galactan, and arabinan are synthesized as a unit on a polyprenyl-P carrier lipid. Subsequent work has provided the experimental basis of what was once speculation. The synthesis of the entire core is initiated on a decaprenyl-P with synthesis of the linker unit, and then there is concomitant extension of the galactan and arabinan chains while this intermediate is transported through the cytoplasmic membrane.[12,13] Some of the enzymatic and genetic details of this complex process have been defined. For instance, the rhamnose of the diglycosyl-P bridge originates in dTDP-rhamnose, and the biosynthesis and genetics of dTDP-rhamnose synthesis and transfer of the rhamnose unit have been defined.[14,15] The Gal*f* units of the galactofuran originate in UDP-Gal*f*, which, in turn, originates in UDP-Gal*p* catalyzed by UDP-Gal*p* mutase.[16] the Rv3808c gene product.[13] The galactosyltransferase responsible for the polymerization of the bulk of the galactofuran has been identified as the Rv3809c gene product.[13] The immediate precursor of the Ara*f* units of the arabinofuran have been identified in the decaprenyl-P-Ara*f*,[17] not a nucleotide precursor. The decaprenyl-P-Ara*f* probably arises in the pentose phosphate pathway.[18,19] The final steps in these events, the attachment of mycolic acids and ligation to PG, await definition and will prove to be excellent drug targets for a new generation of anti-TB drugs.

The arrangement of genes responsible for PG synthesis in *M. tuberculosis* is similar to that in other bacteria, and so is the biochemistry. Hence, the excellent reviews on bacterial PG synthesis, in general,[20,21] are applicable.

One of the greatest triumphs in this area has been the almost complete definition of the genetics and biochemistry of all aspects of fatty acids/mycolic acid synthesis in *M. tuberculosis*. The set of genes, only some of which are transcriptionally coupled, responsible for synthesis of the meromycolate chain of mycolic acids, were defined by C. Barry, R. Slayden, W. Jacobs, M. Schaeffer, and G.S. Besra.[22–27] Another set of genes, four of which are coupled, are responsible for the introduction of chemical modifications into the meromycolate chain, such as methyl, methoxy, and cyclopropane groups. The *fbp* genes are probably responsible for insertion of mycolic acids into the cell wall proper.

Jacobs, Schaeffer, Besra,[22–27] and others have fully defined the biochemical transformations encoded by many of the genes in the mycolic acid synthetic pathway. Acetyl CoA carboxylase (Acc) gives rise to malonyl CoA. FabD exchanges CoA for ACP. FabH is responsible for the coupling of fatty acid synthases I and II (FAS I and FAS II, giving rise to the first precursor of mycolic acid synthesis, a β-ketoacyl-ACP, which then undergoes reduction by MabA, dehydration (the dehydratase has not yet been identified), enoyl ACP reduction catalyzed by inhA, the target of INH, and then another round of elongation catalyzed by KasA and KasB. Schaeffer et al. (personal communication) have already demonstrated the essentiality of several of these enzymes and configured them into high throughput screens for identification of new drugs against TB.

The polyprenyl-P carrier lipid involved in the synthesis of the cell-wall core has now been identified as decaprenyl-P. It is responsible for the synthesis of PG and also for the synthesis of the linker unit—Rha–GlcNAc-P—and subsequently galactan and arabinan formation.[28,29] The carrier lipid is also responsible for the synthesis of polyprenyl-P-linked Ara*f*, the source of arabinan. The carrier lipid may also be involved in the attachment of new mycolic acids to the cell wall. For that reason, the synthesis of the carrier lipid has been worked out by Crick et al.[28,29] It is not synthesized by the mevalonic acid pathway, but by the new non-mevalonate pathway involving deoxyxylulose-5-P, a pathway that provides another good drug target.

Biosynthesis and biological functions of the free lipids of *M. tuberculosis*

Intercalated within the lipid environment provided by the mycolic acids of the mAGP complex are the lipids that have intrigued researchers for over five decades: the phthiocerol dimycocerosate (DIM/PDIM), cord factor/dimycolyltrehalose, the sulfolipids (SLs), the PIMs, etc. Knowledge of their roles in "signaling" events, in pathogenesis, and in the immune response is now emerging. Knowledge of the genome has greatly helped in defining the biosynthesis of these products.

Cord factor/TDM

The most notable biological attribute of cord factor is the characteristic toxicity it exerts in mice; a few repeated intraperitoneal injections of small amounts (10 μg), dissolved in paraffin oil, killed a

majority of animals; however, larger doses (50–100 μg) are rarely lethal. Death is associated with intense peritonitis and acute pulmonary hemorrhage. The biochemical mechanism of cord factor toxicity has been reviewed by Goren and Brennan.[30] Apparently, cord factor stimulates host NADase activity, leading to lower levels of host NAD, especially in lung, liver, and spleen tissues and reduced activities of a range of NAD-dependent microsomal enzymes. According to Kato, cord factor intoxication is attributable to a direct physical effect on mitochondrial membranes, resulting in disruption of electron flow along the mitochondrial respiratory chain and of oxidative phosphorylation. Cord factor, especially when delivered in a variety of formats, is immunogenic, granulomagenic, and adjuvant-active. The antitumor activity of cord factor has also been studied extensively (see Goren and Brennan).[30]

Sulfolipids

Studies by Gangadharan et al. and Goren established a clear correlation between the presence of SLs in *M. tuberculosis* isolates and virulence for guinea pigs.[30] The most virulent strains "were prolific in elaborating strongly acidic lipids (notably SLs), whereas the attenuated ones were notably deficient in these components." Armstrong and Hart had observed that phagosomes containing viable virulent *M. tuberculosis* of mouse peritoneal are resistant to fusion with secondary lysosomes or dense granules, leading to extensive studies by Goren and Hart and the conclusion that sulfolipids were implicated in phagosome–lysome fusion. However, this conclusion was later questioned by Goren himself, leaving in limbo the actual mechanism of SL involvement in disease virulence.[30]

Phthiocerol dimycocerosate

DIM is a major lipid of the tubercle bacillus. When immunologists refer to the tubercle bacillus as a "ball of wax," they are referring to DIM. It is highly apolar. It was discovered by Rudolf Anderson at Yale in the 1940s. It is, in fact, a wax. It has 35 carbons as methyl or methylene groups. It does have two reactive hydroxy groups, but these are esterified with two fatty acids, the mycocerosic acids, every bit as apolar as the phthiocerol itself with 28 methyl or methylene groups. Intriguing older work has also implicated DIM in *M. tuberculosis* virulence. Goren et al. (see Ref.[30]) reported that DIM was found in a wide selection of patient isolates of *M. tuberculosis*, of a wide spectrum of virulence, and was apparently as prominent in highly attenuated strains as in the most virulent ones, including *M. tuberculosis* H37Rv. However, Goren et al. (in Ref.[30]) observed that an attenuated mutant of H37Rv lost the capacity for synthesis of DIM. Goren mentioned that the "biochemical lesion might rest in an inability of the mutant to synthesize mycocerosic acids, phthiocerol," a statement of extraordinary prescience in light of later developments (see below).

DIM and related lipids and methyl branched fatty acids

There has been much recent progress on the biosynthesis of DIM. One of the unexpected features of the *M. tuberculosis* genome was a 50-kB fragment containing 13 genes which had the hallmarks of a type-I modular polyketide synthase, which initially left researchers puzzled, since the chemists had not shown any evidence of classical polyketides in *M. tuberculosis*. However, in the midst of this operon, transcribed in the other direction, is a gene, *mas*, mycocerosic acid synthase, and Rainwater and Kolattukudy,[31] Azad et al.,[32] Fernandes and Kolattukudy,[33] and Fitzmaurice and Kolattukudy[34] had shown that this encoded an enzyme with a subunit molecular weight of 238,000, an iterative polyketide synthase, that acts like a FAS I, but produces mycocerosic acid after four rounds of extension of a C_{18} fatty acid but using methyl malonyl CoA instead of malonyl CoA, which is the source of the methyl branches of mycocerosic acid.

This early work by Kolattukudy et al. suggested that the other genes of the cluster were responsible for phthiocerol synthesis, again through a modular polyketide synthase mechanism, and they provided evidence to this effect. A FAS I-like system synthesizes a straight-chain fatty acyl group attached to its enzyme. Then module I (Pps1) contains an acyl transferase domain, a ketoacyl synthase domain and a keto reductase domain, allowing introduction of the hydroxyl group. Pps2 does the same thing. However, Pps 4 and 5 use methylmalonyl CoA, allowing the introduction of the characteristic methyl branches of phthiocerol. Finally, there are reduction and decarboxylation steps to produce the phthiocerol, and the acyl CoA synthase (FadD28) responsible for the attachment of mycocerosic acids, synthesized by Mas, to the phthiocerol synthesized by the Pps modules, has been identified.

Cox et al.[35] and Camacho et al.[36] have recently begun to address the role of DIM in disease

pathogenesis. Using signature-tagged transposon mutagenesis, Cox et al.[35] isolated mutants with insertions upstream of *fadD26* in the *pps* region and within *fadD28* and *mmpl* 7. The French group[36] isolated mutants with insertions upstream from *fadD26* and within *fad26* and within *drrC* and *mmpl* 7. The results from both laboratories were similar and were intriguing. No DIM production or production of a related lipid was seen in the upstream *fadD26*, i.e., *pps* mutant or the *fadD28* mutant. However, the *mmpl* 7 mutant could synthesize DIM, but it could not excrete DIM into the medium: it and a *drrC* mutant were defective in DIM export/excretion.

Already some interesting biology has been done on the DIM-less mutants.[35,36] They show attenuated growth in the mouse lung. They also show much higher cell-wall permeability, all fitting in with old work from Goren and Brennan (see Ref.[30]) that a DIM-less variant of *M. tuberculosis* H37Rv showed attenuated growth in the guinea pig.

Reports have also recently come from Kolattukudy's lab on the generation of mutants devoid of SLs.[37] These are trehalose derivatives, largely defined by Goren, but with a sulfate group at the 2 position of trehalose (see Ref.[30]). They also have methyl-branched fatty acids, called the phthioceranic or hydroxy-phthioceranic acids. A *mas*-like *pks2* gene responsible for their synthesis was identified which was successfully disrupting by homologous recombination.[37] In light of the controversial role of SLs in TB pathogenesis, it will be intriguing in the future to see the phenotype of these mutants.

PIMs, LM, and LAM

Another group of free lipids that are benefiting from the genome in terms of biosynthesis and function are the PIMs, LM, and LAM.

The PIMs themselves were described by C.E. Ballou and colleagues in the 1960s (see Ref.[30]). They are based on phosphatidylinositol (PI) and attached to the inositol there may be from one to six mannoses. The PIMs with two mannoses are the most common in *M. tuberculosis*. Some years ago, we discovered that molecules described as LM, lipomannan, and LAM, lipoarabinomannan, are extensions of PIM, in that, in the case of LM, the mannan chain was extended and branching was introduced; in the case of LAM, an additional arabinan is attached.[38]

Chatterjee and Khoo[38] and Puzo and colleagues[39] are mostly responsible for the modern-day structure of LAM.

There is intense interest in the role of LAM in TB. LAM from *M. tuberculosis* has short mannose-containing oligosaccharide "caps" that allow it to bind to the mannose receptor on macrophages, unlike the product from *M. smegmatis*, which has no such mannose caps. Also, LAM can bind to Toll receptors and can physically insert itself into membranes, inducing all sorts of signaling events important in the host response in TB. Thus, understanding the biosynthesis of LAM and the creation of LAM-less mutants are major priorities.

Based on structures, one would predict that the first event would be synthesis of PI, then the addition of mannose to give PIM_1, PIM_2, and so on. However, in the original genome sequence, the genes for phosphatidylserine and phosphatidylethanolamine synthesis were clearly annotated. However, all other genes for phospholipid synthesis, including the one that may be responsible for PI synthesis, could not be distinguished, because all of them use CDP-diacylglycerol as the source of diglyceride, and its binding motif dominated the structures of the genes. Hence, these were named as *pgsA*, $pgsA_2$, $pgsA_3$. M. Jackson from Dr. B. Gicquel's laboratory, working initially with us and then back home, overexpressed each one of these in *M. smegmatis* and established that only *pgsA* (Rv2611c) was responsible for the synthesis of PI.[40] PgsA is part of a small operon containing Rv2609c, Rv2610c, and Rv2611c. Jackson et al.[41] have now shown that *pimA* (Rv2610c) adds on the first mannose to PI, the Rv2611c then probably adds on a fatty acid. Previously, Schaeffer et al. had shown that PimB, half a chromosome away, adds on the second mannose.[42] So, pgsA, the PI synthase, allows the condensation of inositol and the diglyceride of the CDP-diacylglycerol derivative. PimA allows the addition of mannose 1 followed by an acyl function. PimB allows the addition of the second mannose. Gurcha et al.[43] recently described a PimC that attaches the third mannose; they speculated that this is the direct precursor of LM and LAM.

Even though the early genes in the PIM/LAM biosynthetic pathway are essential and required for growth, it should now be possible to generate LAM-less mutants in order to explore the biological role of LAM.

Acknowledgements

Research in the author's laboratory was supported by grants AI-18357 and AI-46393 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

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